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Interaction of Parvovirus B19 with Human Erythrocytes Alters Virus Structure and Cell Membrane Integrity▽

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The unique region of the capsid protein VP1 (VP1u) of B19 virus (B19V) elicits a dominant immune response and has a phospholipase A_2 (PLA_2) activity required for the infection. Despite these properties, we have observed that the VP1u-PLA_2 motif occupies an internal position in the capsid. However, brief exposure to increasing temperatures induced a progressive accessibility of the PLA_2 motif as well as a proportional increase of the PLA_2 activity. Similarly, upon binding on human red blood cells (RBCs), a proportion of the capsids externalized the VP1u-PLA_2 motif. Incubation of B19V with RBCs from 17 healthy donors resulted in extensive virus attachment ranging between 3,000 and 30,000 virions per cell. B19V empty capsids represent an important fraction of the viral particles circulating in the blood (30 to 40%) and bind to RBCs in the same way as full capsids. The extensive B19V binding to RBCs did not cause direct hemolysis but an increased osmotic fragility of the cells by a mechanism involving the PLA_2 activity of the exposed VP1u. Analysis of a blood sample from an individual with a recent B19V infection revealed that, at this particular moment of the infection, the virions circulating in the blood were mostly associated to the RBC fraction. However, the RBC-bound B19V was not able to infect susceptible cells. These observations indicate that RBCs play a significant role during B19V infection by triggering the exposure of the immunodominant VP1u including its PLA_2 constituent. On the other hand, the early exposure of VP1u might facilitate viral internalization and/or uncoating in target cells.

Human parvovirus B19 (B19 virus; B19V) was discovered in 1975 (7), and it has been classified within the *Erythrovirus* genus of the *Parvoviridae* family. B19V is generally associated with a mild and frequent childhood disease named erythema infectiosum, or fifth disease (1). However, in certain situations or in individuals with altered immunologic or hematologic conditions, B19V can cause other more severe syndromes such as acute and chronic arthropathies (28), hemolytic disorders (32), hydrops fetalis, and fetal death (5, 10).

The single-stranded DNA genome of B19V is packaged into a small nonenveloped, icosahedral capsid consisting of 60 structural subunits, of which approximately 95% are VP2 (58 kDa) and 5% are VP1 (83 kDa). VP1 and VP2 originate from overlapping reading frames and are identical except for a stretch of 227 additional amino acids at the VP1 N-terminal region, the so-called VP1 "unique region" (VP1u) (9, 26). Even though this region is a minor component of the capsid, the dominant immune response against B19V is elicited by the VP1u region, which harbors strong neutralizing epitopes (2, 31, 45). A poor immune response against VP1u has been linked to persistent infections (21). Apart from its immunodominant role, VP1u harbors a phospholipase A_2 (PLA_2) motif (13), which is required for the infection (14, 18, 44). Growing evidence indicates that VP1u also plays a central role in the induction of autoimmune reactions and inflammatory processes (22, 36, 37, 41) by mechanisms still not well understood. Despite all these properties, we have recently shown that the most N-terminal part of VP1u harboring strong neutralizing epitopes (2) is not external to the capsid. However, brief exposure to mild temperatures or low pH rendered this region accessible and triggered the VP1u-PLA_2 activity of the virus (30). B19V would therefore be similar to other parvoviruses in which the internal VP1u can become exposed in vitro by treatments with mild heat or low pH (3, 8, 14, 20, 34, 39) and in vivo during the intracellular trafficking of the virus (24, 29, 33, 39). Given that the VP1u of B19V is the immunodominant constituent of the capsid, it is reasonable to expect that this region should become exposed not during the intracellular trafficking of the virus but already in the extracellular milieu. In this sense, it is tempting to speculate that binding of B19V to receptors on the cell surface would act as the trigger to render VP1u accessible. It has been previously shown for several viruses that binding to the cellular receptor can trigger changes in capsid conformation (27). Upon attachment to susceptible cells, polioviruses, rhinoviruses, and most related enteroviruses undergo conformational transitions that alter the accessibility of several regions (11, 15). Such changes have not yet been observed in parvoviruses.

The cellular receptor of B19V is globoside (4), which is necessary but not sufficient for the infection (42). Other required coreceptors have been identified such as the α5β1 integrin and Ku80 (25, 40). The coreceptor α5β1 integrin is thought to be required for internalization (40). In order to be fully immunogenic, VP1u would have to be stably exposed on the cell surface without subsequent virus internalization. B19V binding without internalization might occur in different cell types lacking the required conditions. Mature human red blood cells (RBCs) are one of these types of cells. They are

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very abundant, express large quantities of the B19V receptor globoside (16), but are unable to internalize B19V (40). Moreover, B19V does not bind membrane-associated globoside in vitro (19), indicating that B19V probably binds globoside in concert with other molecular structures present on cell membranes. Even though the RBCs are major viral targets, particularly during the viremic phase of the infection when large amounts of infectious viral particles circulate in the blood, the interaction of B19V with the RBCs has not yet been investigated.

MATERIALS AND METHODS

Cells and viruses. UT7/Epo cells were cultured in RPMI medium with 10% fetal calf serum, and 2 U/ml of recombinant human erythropoietin (Epo; Janssen-Cilag, Midrand, South Africa) at 37°C and 7.5% CO_2. Heparinized blood samples were obtained from healthy donors. The RBCs were washed four times with phosphate-buffered saline (PBS) before use. A blood sample was obtained from an individual with a recent B19V infection (immunoglobulin M [IgM] and IgG positive). A B19V-infected plasma sample was obtained from our donation center (genotype 1; CSL Behring AG, Charlotte, NC). The viremic serum sample did not contain B19V-specific IgM or IgG antibodies. B19V was concentrated from the infected serum by ultracentrifugation through 20% sucrose. The viral pellets were washed and resuspended in PBS. B19V titers were determined by quantitative PCR as DNA-containing particles per microliter. Gradient-purified B19V full capsids (FC) and empty capsids (EC) were prepared from the viremic serum sample as previously described (23). The amounts of FC and EC were examined by dot blot hybridization with a mouse antibody against B19V viral proteins (1:500; US Biologicals, Swampscott, MA), followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution). Additionally, the amount of FC as well as the purity of EC was examined by quantitative PCR.

Antibodies. A synthetic peptide derived from the PLA_2 motif of B19V (amino acids [aa] 142 to 163) was used to immunize rabbits (Fig. 1A). This peptide was chosen because it was previously shown to induce neutralizing antibodies (2). The peptide was synthesized as a multiple antigenic peptide, built on a tetrabranched lysine core with a C-terminally amidated cysteine residue for coupling purposes. Peptide-specific antibodies were affinity purified. Peptide synthesis, immunization, and affinity purification were performed by ImmunoGlobe (Himmelstadt, Germany). Two monoclonal antibodies (MAbs), one specific for the most N-terminal region of VP1u (MAb 1418-1) and the other recognizing only intact capsids (MAb 860-55D), were kindly provided by S. Modrow (Regensburg, Germany). The two MAbs were produced from peripheral blood mononuclear cells from healthy individuals with high titers of serum antibodies against B19 virus proteins (17). A globoside-specific IgM monoclonal antibody (AME-2) (40) was kindly provided by J. de Jong (The Netherlands Red Cross, Amsterdam, The Netherlands). A mouse nonspecific IgM isotype control was purchased from Sigma (St. Louis, MO).

Quantitative PCR. Amplification of B19V DNA and real-time detection of PCR products were performed by using a LightCycler system (Roche Diagnostics, Rotkreuz, Switzerland) with Sybr Green (Roche). PCR was carried out using a FastStart DNA Sybr Green kit (Roche) following the manufacturer's instructions. Primers used for B19V-DNA amplification were B19V-forward (5′-GGGCAGCCATTTTAAGTGTTT-3′) and B19V-reverse (5′-GCACCACC AGTTATCGTTAGC-3′). As external standards, plasmids containing the genome of B19V were used in 10-fold dilutions.

Analysis of B19V binding to human RBCs. Binding of B19V to RBCs was examined by quantitative PCR. RBCs from a total of 17 individuals were washed with PBS and resuspended at a concentration of 2×10^7 RBCs per ml. A volume of 10 μl of the RBC suspension was mixed with B19V (2×10^5 virions per cell). After 2 h at 4°C the cell/virus suspension was washed six times with PBS to remove unbound virus. The cell pellet was resuspended in 200 μl of PBS, and a fraction was used to count the cells. Total DNA was extracted from the cell suspension by using a DNeasy tissue kit (Qiagen, Valencia, CA). RBC-associated viral DNA was quantified by using real-time PCR as specified above. The results were compared with values obtained from corresponding amounts of RBCs spiked with increasing amounts of B19V.

Additionally, the RBCs were incubated with a mouse IgM antibody specific for the cellular receptor globoside (AME-2) (40), with a similar concentration of a nonspecific mouse IgM isotype control antibody (Sigma) or with EC (5×10^4 particles per cell). After 1 h at 4°C, the cells were washed, and B19V was added (2.5×10^4 virions per cell) and further incubated at 4°C for 2 h.

Binding of B19V EC to RBCs was examined by immunofluorescence. RBCs

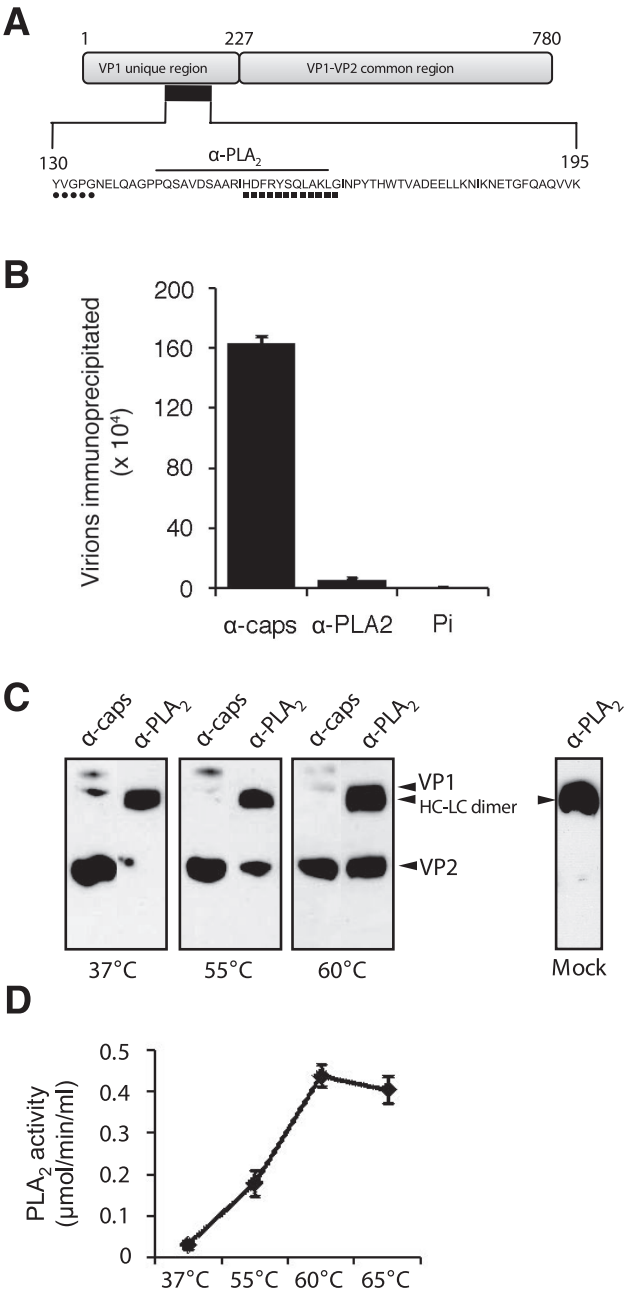

FIG. 1. Accessibility of the PLA_2 region of B19V VP1u. (A) Schematic representation of the VP1/VP2 proteins of B19V. Filled circles indicate the calcium binding domain, and filled squares indicate the PLA_2 enzymatic core. The sequence of the peptide used for the immunization of rabbits is indicated. (B) Accessibility of the VP1u-PLA_2 motif in native B19V capsids. B19V DNA was quantified following immunoprecipitation with an antibody against capsids (α-caps; MAb 860-55D) or an antibody against the PLA_2 region (α-PLA_2; VP1u 142–163). (C) Accessibility of the VP1u-PLA_2 region after heat treatment. B19V capsids were treated at increasing temperatures for 3 min, cooled on ice, and immunoprecipitated with MAb 860-55D (α-caps) or VP1u 142–163 (α-PLA_2). The 75-kDa band corresponds to an LC-HC dimer resulting from partial denaturation of the antibody (band present without virus). (D) Activation of B19V-PLA_2 enzyme by heat treatment. PLA_2 activity of B19V capsids was measured following exposure to increasing temperatures for 3 min. Error bars represent the deviation from three independent experiments.

were incubated with FC or EC as specified above, and the cells were fixed with acetone-MeOH (1/1, vol/vol) for 5 min at −20°C. After fixation, the cells were air dried and washed with PBS—1% bovine serum albumin (PBSA). Subsequently, a human MAb recognizing only intact capsids (MAb 860-55D) was applied (diluted 1:50 in PBSA) for 1 h at room temperature. The cells were washed three times with PBSA for 5 min, and as a secondary antibody, goat anti-human IgG-fluorescein isothiocyanate was added (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After final washings with PBSA, the cells were mounted with mowiol (Calbiochem, La Jolla, CA) containing 30 mg/ml of Dabco (Sigma) as an antifading agent and examined by fluorescence microscopy.

Infectivity assay. B19V particles were bound to RBCs as specified above. Following binding, the cells were washed to remove unbound particles. The amount of virus bound per cell was quantified as previously described. Subsequently, the RBCs were added as intact or lysed cells to UT7/Epo cells (10^5 cells) at 1,000 B19V DNA-containing particles per cell in RPMI medium without serum. As controls, similar amounts of free B19V without RBCs or in the presence of RBCs that were preblocked with EC were used. After 1 h at 4°C, the cells were washed three times in PBS and transferred to 12-well plates and further incubated at 37°C in RPMI medium containing 10% fetal calf serum and Epo. For viral RNA analysis, the cells were transferred after 24 h to RNase-free tubes (1.5-ml Safe-Lock Tubes; Eppendorf Biopur) and pelleted. The pellet was washed twice with PBS and stored at −20°C until use. Total poly(A) mRNA was isolated with an mRNA isolation kit (Roche). Following reverse transcription, cDNA was quantified by using a LightCycler 2.0 system (Roche) and the LightCycler FastStart DNA Master Sybr Green I kit (Roche). Primers were chosen to amplify a 133 nucleotide (nt)-long NS1 cDNA fragment: NS1 forward (5′-GGGGCAGCATGTGTTAAAG-3′ (nt 1017 to 1035) and NS1 reverse (5′-CCATGCCATATACTGGAACACT-3′ (nt 1129 to 1150). For viral DNA analysis, the cells were collected 84 h postinfection. Total DNA was extracted, and viral DNA was quantified as specified above.

Immunoprecipitation. B19V capsids were immunoprecipitated with a human MAb against intact capsids (MAb 850-55D) or with a rabbit polyclonal antibody against the PLA_2 region (VP1u residues 142 to 163 [VP1u 142–163]). After overnight incubation at 4°C in the presence of 20 μl of protein G agarose beads, the supernatant was discarded. The beads were washed four times with PBSA. Immunoprecipitated viral capsids were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. After transfer to a polyvinylidene difluoride membrane, the blot was probed with a mouse antibody against B19V proteins (1:500; US Biologicals, Swampscott, MA), followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution). The viral structural proteins were visualized with a chemiluminescence system (Pierce, Rockford, IL). Immunoprecipitation of B19V bound to RBCs was performed after cell lysis in NP-40 buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% NP-40, pH 7.2). Following centrifugation at 10,000 × *g* for 10 min, the supernatant was used for immunoprecipitation. Quantification of the immunoprecipitated virions was performed by real-time PCR as specified above.

PLA_2 activity assay. Native B19V capsids were assayed for PLA_2 activity by using a colorimetric assay ($sPLA_2$ assay kit; Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions. The absorbance at 414 nm was determined every minute. The PLA_2 activity was expressed as micromoles of substrate hydrolyzed per minute per milliliter.

OF test. The osmotic fragility (OF) test measures RBC resistance to hemolysis when cells are exposed to hypotonic saline solutions. An increased OF indicates damage or alteration of the RBCs. RBCs (2×10^6) were incubated with increasing amounts of B19V per cell in a final volume of 10 μl of PBS. Following virus attachment for 1 h at 4°C, the cells were incubated in a buffer containing calcium (20 mM HEPES, 150 mM NaCl, 10 mM $CaCl_2$, 0.5 μg/ml bovine serum albumin) for 3 h at 37°C. After centrifugation, the amount of hemoglobin of each supernatant was measured in a spectrophotometer at 545 nm. The results were expressed as the percentage of hemolysis compared with the absorbance obtained after total cell lysis (corresponding to the amount of RBCs in water); this is referred to as direct hemolysis. Subsequently, the RBC pellets were resuspended in a hypotonic NaCl buffer (0.6g/dl) and incubated overnight at 37°C. The percentage of hemoglobin release was measured in the same way and is referred to as OF.

RESULTS

The PLA_2 motif of native B19V capsids is not accessible to antibody binding but can become exposed and active upon mild heat treatment. We have previously found that the PLA_2 activity is barely detectable in the native B19V capsids but can be triggered upon treatment with mild heat or low pH (30). The question remained whether the lack of activity was due to an internal position within the capsid. In the present studies, we have verified the accessibility of this region by using an antibody (VP1u 142–163) targeting the PLA_2 enzymatic region (Fig. 1A). The results showed that the PLA_2 region of B19V is internal and not accessible to antibodies (Fig. 1B). Treatment of the native capsids to increasing temperatures induced a progressive accessibility of the PLA_2 motif (Fig. 1C) as well as a proportional increase of PLA_2 activity (Fig. 1D). Therefore, in order to be active, the PLA_2 region of B19V has to become fully exposed in a conformation which is accessible to antibody binding.

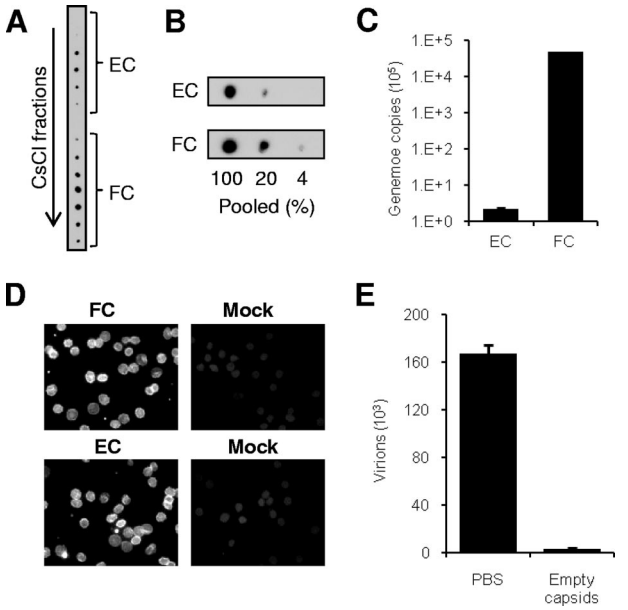

FIG. 2. Proportion of FC and EC from an infected plasma sample. Dot blot hybridizations from CsCl fractions (A) and from dilutions of the pooled fractions (B) are shown. (C) Quantification of B19V-DNA from FC and EC pool fractions. (D) Detection of FC and EC bound to RBCs by immunofluorescence. (E) RBCs were incubated with EC (5×10^4 particles per cell). After 1 h at 4°C, the cells were washed, and B19V was added and further incubated at 4°C for 2 h. Following intensive washing to remove unbound virus, the amount of B19V associated to RBCs was quantified by real-time PCR. Deviations from three independent experiments are indicated.

B19V EC represent an important fraction of the viral particles circulating in the blood and bind RBCs in the same way as FC. For some animal parvoviruses, it has been shown that during the infection process in cell culture DNA-containing capsids (FC) and EC are generated. We have examined the proportion of FC and EC from a naturally infected individual. Gradient-purified FC and EC were prepared from a viremic serum sample. A dot blot hybridization performed with the CsCl fractions showed the separation of EC and FC (Fig. 2A). The same experiment performed with pool fractions showed that the EC represent an important proportion (estimated at 30 to 40%) of the B19V particles circulating in the blood (Fig. 2B). The purity of the EC as well as the concentration of FC was verified by quantitative PCR (Fig. 2C).

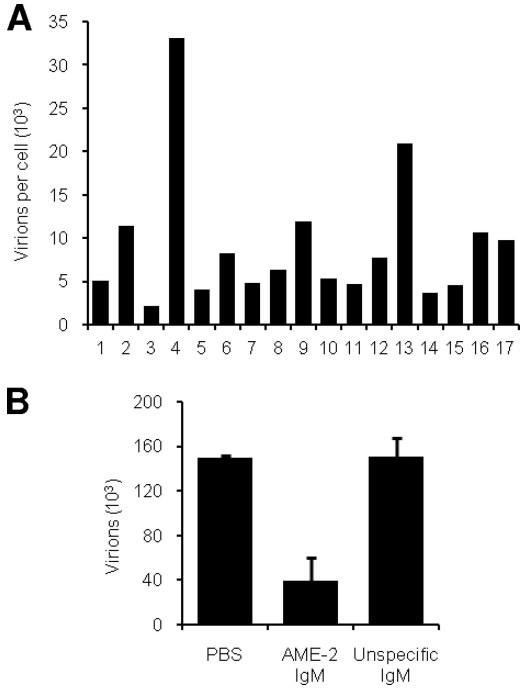

FIG. 3. (A) Quantification of B19V binding to RBCs from different donors. B19V was incubated with RBCs from 17 healthy donors. Following washings to remove unbound virus, the amount of virus bound per cell was quantified by real-time PCR. (B) Effect of pretreatment of RBCs with an antibody specific for globoside on B19V binding. RBCs where either untreated (PBS) or treated with anti-globoside IgM (AME-2) or a similar concentration of a nonspecific mouse IgM isotype control before the addition of B19V (2.5×10^4 virions per cell). Deviations from three independent experiments are indicated.

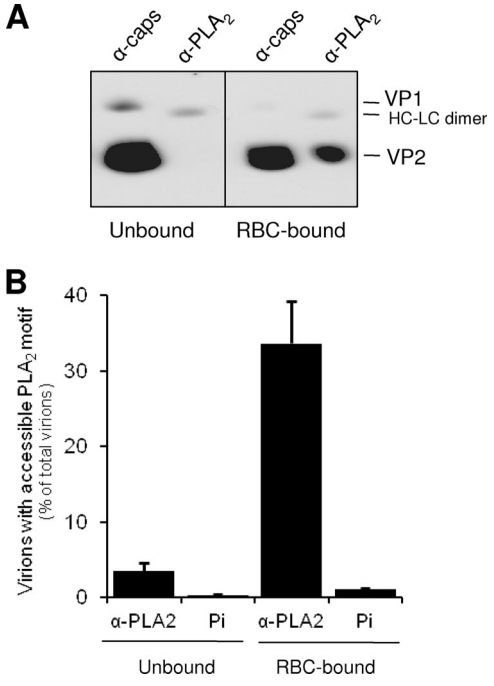

FIG. 4. VP1u-PLA_2 is exposed on the capsid surface following attachment to P antigen on human RBCs. (A) Following binding to RBCs at 4°C, the supernatant (unbound virus) and cellular fractions (bound virus) were incubated in lysis buffer and centrifuged at $10,000 \times g$ for 10 min. The viral capsids present in the supernatants were immunoprecipitated with a MAb against assembled capsids (α-caps; 860-55D) and a polyclonal antibody against the VP1u-PLA_2 motif (α-PLA_2; VP1u 142–163). The amount of virus present in the immunoprecipitated material was examined by Western blotting. (B) The amounts of virions immunoprecipitated with the antibody against capsids, with the antibody against the PLA_2 motif, or with the preimmune serum from the rabbits used to produce the PLA_2 antibody were quantified by real-time PCR. Error bars represent the deviations from four independent experiments.

Given the important proportion of B19 EC in the viremic serum, it was of interest to verify the attachment of EC to RBCs. The immunofluorescence experiments showed that EC also bind to RBCs (Fig. 2D). Preincubation of RBCs with pure B19V EC resulted in a total binding inhibition of FC (Fig. 2E), indicating that both FC and EC bind to RBCs through the same receptor structures.

Quantification of B19V binding to RBCs from different donors. Incubation of B19V with RBCs from 17 healthy donors resulted in extensive virus attachment ranging between 3,000 and 30,000 virions per cell. Most of the samples showed a binding rate of approximately 4,000 to 12,000 virions per cell. Two of the 17 samples had a higher binding capacity, reaching 20,000 to 30,000 virions per cell (Fig. 3A).

B19V binding to RBCs can be disturbed by an antibody specific for globoside. It has been previously shown that binding of B19V to susceptible cells involves other receptors apart from globoside, namely, Ku80 and α5β1 integrin (25, 43). Human RBCs have globoside but lack integrin (43). We have investigated the blocking effect of a MAb specific for globoside (AME-2) (40) on the binding efficiency of B19V to RBCs. Preincubation of RBCs with AME-2 disturbed binding of B19V to RBCs (Fig. 3B). This result indicates that globoside is involved in the binding of B19V to RBCs. However, since the blocking effect is not complete, other molecular structures might also be involved in the binding.

Binding of native B19V to RBCs triggers the exposure of the VP1u-PLA_2 motif. We have previously shown that the most N-terminal part of VP1u, containing neutralizing epitopes, is not exposed on the surface of native B19V particles (30). In the present studies we also show that the PLA_2 region, distant from the N-terminal part, is also not accessible to antibodies. Given the fact that VP1u contains critical neutralizing epitopes, it should become accessible to antibodies in the extracellular milieu. In view of that, the possibility that binding of B19V to human RBCs would trigger the exposure of VP1u was investigated.

Following binding to RBCs at 4°C, the viral capsids present in the supernatant (unbound virus) and cellular fractions (bound virus) were immunoprecipitated with a MAb against assembled capsids (MAb 860-55D) and a polyclonal antibody against the VP1u-PLA_2 motif (VP1u 142–163). The amount of virus present in the immunoprecipitated material was examined by Western blotting. As shown in Fig. 4A, viral capsids with an exposed VP1u-PLA_2 motif were detected only in the cell fraction. From the immunoblotting results, it was evident that VP1u was not exposed in all capsids bound to RBCs. In order to quantify the proportion of virions with the VP1u-PLA_2 motif exposed, the viral DNA present in the immuno-

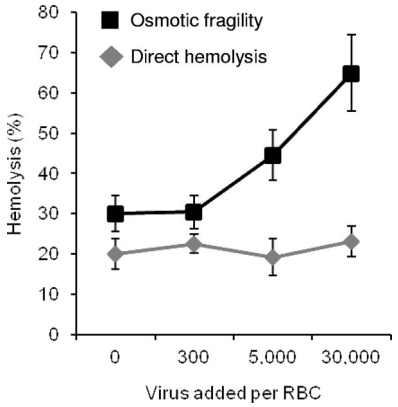

FIG. 5. B19V increases the OF of human RBCs. RBCs (2×10^6) were incubated with increasing amounts of B19V per cell (virus concentrated from serum by ultracentrifugation through 20% sucrose and resuspended in PBS). Following virus attachment for 1 h at 4°C, the cell suspension was incubated in an isotonic buffer containing calcium for 3 h at 37°C. The percentage of hemoglobin release was measured in the supernatant and referred to as direct hemolysis. Subsequently, the RBC pellet was resuspended in a hypotonic NaCl buffer (0.6g/dl) and incubated overnight at 37°C. The percentage of hemoglobin release was measured in the same way and was referred to as OF. Results are mean values from three independent experiments.

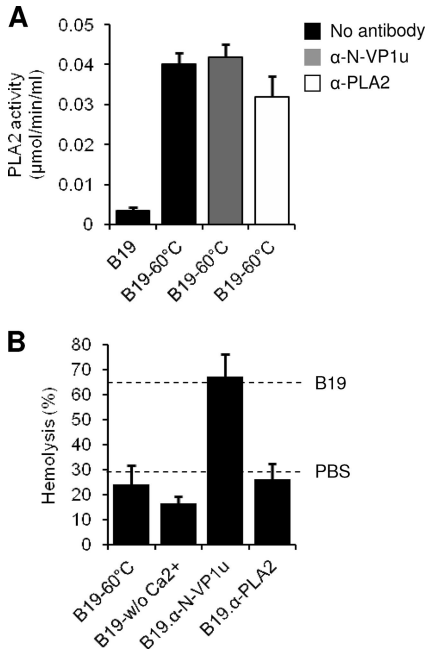

FIG. 6. The VP1u-associated PLA_2 activity is responsible for the increased OF of human RBCs. (A) B19V-PLA_2 activity in the presence of antibodies against VP1u. The PLA_2 activity from heat-treated (60°C for 3 min) B19V (1.2 μg) was measured after incubation with MAb 1418-1 (α-N-VP1u; against the most N-terminal part of VP1u) or with antibody VP1u 142–163 (α-PLA_2; targeting the PLA_2 region). (B) OF of RBCs incubated with B19V under different conditions. RBCs (2×10^6) were incubated with 3×10^4 virions per cell. Following virus attachment for 1 h at 4°C, the cells were incubated in an isotonic buffer with or without calcium or in the presence of antibodies against VP1u for 3 h at 37°C. Following centrifugation, the RBC pellets were resuspended in a hypotonic NaCl buffer (0.6g/dl) and incubated overnight at 37°C. The percentage of hemoglobin release was measured. The graph indicates the average OF value of RBCs incubated with B19V (upper line) or without (w/o) B19V (lower line). B19, B19V; B19-60°C, B19V exposed to heat treatment of 60°C for 3 min. Error bars from three independent experiments are indicated.

precipitated material was quantified by real-time PCR. Following binding to RBCs, the proportion of virions that changed conformation and exposed VP1u-PLA_2 was approximately one-third of the total number of particles (Fig. 4B). Therefore, there are capsids bound to RBCs that did not exposed VP1u. This result might be due to the observation that binding of B19V to RBCs is complex and might involve various molecular structures, which would have a different effect on the conformation of VP1u.

B19V is not directly hemolytic but increases the OF of RBCs. Binding of B19V to human RBCs results in the exposure of the PLA_2 motif present in the VP1u. Treatment of human RBCs with PLA_2 from bee venom does not cause direct hemolysis but increases the OF in parallel with the cleavage of the accessible phospholipids (38). Since B19V binds extensively to human RBCs without internalization, it was of interest to examine whether the exposed PLA_2 enzyme affects the integrity of the RBC membranes. Similar to the PLA_2 from bee venom, no direct hemolysis was observed after 3 h of incubation at 37°C with B19V capsids; however, the OF of the RBCs increased progressively with the number of viruses per cell (Fig. 5), indicating a damage or an alteration in the cell membranes.

Inhibition of the PLA_2 activity of B19V capsids. The PLA_2 activity of B19V capsids was assessed in the presence of antibodies against VP1u. The antibody 1418-1 (N-VP1u), which targets a region of VP1u (aa 30 to 42) distant from the PLA_2 motif (12), had no effect on the enzyme activity. The antibody against the PLA_2 region only moderately inhibited the activity (Fig. 6A). This result indicates that the antibody does not bind any critical amino acid required for the enzymatic activity with a soluble substrate.

The VP1u-associated PLA_2 activity is responsible for the increase in the OF of RBCs. In order to investigate whether the B19V-PLA_2 activity is responsible for the increase in the OF of the RBCs, the OF test was performed in the absence of calcium, which is required for PLA_2 activity (6), or in the presence of antibodies against VP1u (1 μg). The MAb against the most N-terminal part of VP1u (aa 30 to 42; MAb 1418-1), which is distant from the PLA_2 motif, had no influence on the increase in OF caused by B19V. However, under calcium depletion or in the presence of the PLA_2 antibody, B19V was not able to modify the OF of the RBCs (Fig. 6B). Heat-treated capsids (at 60°C for 3 min), which were previously shown to expose the PLA_2 motif (Fig. 1C and D), did not cause any detectable increase in OF, indicating that the PLA_2-phospholipid interaction depends on a specific conformation of the capsid-receptor complex, which might be different in the heat-treated virus. None of the conditions tested, such as calcium depletion, VP1u antibodies, or mild heat treatment, disturbed B19V binding to RBCs or modified the OF of the RBCs in the absence of the virus (data not shown).

Quantification of B19V from plasma and RBC fractions from an individual with a recent infection. Even though RBCs have large amounts of the globoside receptor, B19V viremia is

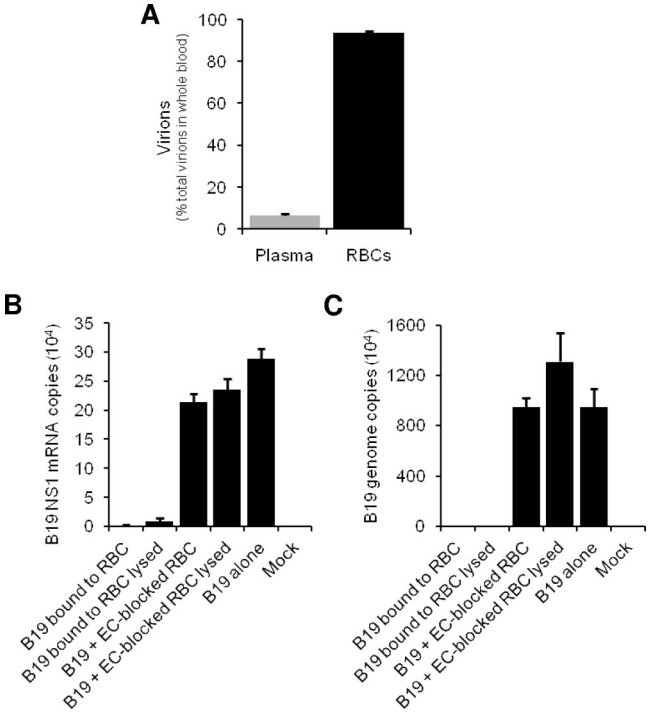

FIG. 7. (A) Quantification of B19V in the plasma and RBC fractions from an individual with a recent B19V (B19) infection (IgG and IgM positive). The heparinized fresh blood sample was centrifuged to separate plasma and RBCs. The RBCs were extensively washed in PBS. B19V DNA was quantified from a volume of 100 μl of plasma or washed RBCs, as specified in Materials and Methods. Infectivity of B19V bound to human RBCs based on NS1 RNA (B) or viral DNA quantification (C) is shown. RBCs were preincubated with B19V. Subsequently, the RBCs were washed intensively with PBS to remove unbound virus. The RBCs were added as intact (in PBS) or lysed cells (in water) to UT7/Epo cells. As controls, similar amounts of B19V alone or in the presence of RBCs that were preblocked with EC were used. NS1 RNA and viral DNA were quantified after 24 h and 84 h, respectively. Error bars from three independent experiments are indicated.

always examined in the plasma fraction while the RBC fraction is ignored. The amount of B19V in the plasma and RBC fractions of a blood sample from an individual with a recent B19V infection (IgG and IgM positive) was examined by quantitative PCR. In plasma and RBC fractions, the amounts of B19V DNA were 1.7×10^7 and 2.7×10^8 virions/ml, respectively. This result indicates that in this particular individual and at this particular moment of the infection, more than 90% of the virions circulating in the blood were associated to RBCs (Fig. 7A).

B19V bound to RBCs cannot infect UT7/Epo cells. Given the fact that B19V particles circulating in the blood can be associated to RBCs, it was of interest to investigate whether such particles are still able to infect a susceptible cell line. Following binding of B19V to RBCs, the cells were extensively washed to remove unbound virus. The RBCs were added as intact or lysed cells to UT7/Epo cells. B19V infectivity was measured by NS1 mRNA and viral DNA quantification, as specified in Materials and Methods. The results showed that viruses attached to RBCs are not able to initiate an active infection in UT7/Epo cells, most probably due to the firm attachment of the virus to the RBCs (Fig. 7B and C).

DISCUSSION

Human RBCs are the most abundant cell type in the blood. Despite the fact that RBCs have large quantities of the B19V receptor globoside (16) and that B19V viremia is typically high, the interaction between B19V and RBCs has not yet been studied. Our observations indicate that B19V interacts extensively with human RBCs. This important interaction results in changes in virus structure and membrane integrity.

The VP1u region is immunodominant and has the PLA_2 activity (13) that is required for the infection (14, 18, 44). VP1u is also suspected to contribute to autoimmune reactions and inflammatory processes by mechanisms still not well understood. VP1u has been shown to generate the production of anti-phospholipid antibodies and anti-phospholipid syndrome-like autoimmunity via molecular mimicry (36, 37). The PLA_2 activity of VP1u is also suspected of contributing to inflammatory processes induced by the production of potent eicosanoid lipid mediators (22). All of these immunological features contrast with our recent findings indicating that the most N-terminal part of VP1u is internal and that the capsids are enzymatically inactive (30). In the present studies we have analyzed the accessibility of the PLA_2 region of VP1u, which is close to the VP1-VP2 junction, and found that this region of VP1u is also internal in native capsids. It is well known for some viruses that binding to their cellular receptors leads to capsid structural rearrangements required for entry into susceptible cells. When poliovirus attaches to its receptor, the particle changes in conformation, and internal components, including VP4 and the N terminus of VP1, are externalized (27, 35). In the case of parvovirus, changes occurring during virus binding to cellular receptors have not been observed so far. In the present studies we have observed changes in capsid conformation following B19V binding to RBCs, leading to the externalization of VP1u sequences. The significance of the exposure of the VP1u sequences at the cell surface remains unknown. It is tempting to speculate that the early exposure of VP1u on the membrane of target cells is followed by interaction with a coreceptor, which would facilitate virus entry. In line with this hypothesis is the fact that globoside is necessary but not sufficient for B19V infection (42). Other coreceptors have been identified such as Ku80 and $\alpha5\beta1$ integrin (25, 43). The role of these coreceptors in B19V infection is still not clear, but it has been postulated that $\alpha5\beta1$ integrin is required for viral entry. Therefore, the externalization of VP1u sequences at the cell surface would facilitate entry and/or uncoating of B19V. The exposure of VP1u sequences at the cell surface would also have implications in the immunology of B19V infection. Antibodies derived from the exposed VP1u sequences would not target and neutralize free infectious particles circulating in the blood, whose VP1u epitopes are not accessible, but would instead target receptor-attached virus. This mechanism of neutralization at the cell surface has already been observed for MAb 1418-1, which targets the most N-terminal part of VP1u. This antibody is highly neutralizing (17) but is not able to bind B19V in a cell-free system (30).

The exposure of VP1u has an impact on the red cell mem-

brane. Similar to the effect observed during treatment of red cells with bee venom PLA_2 (38), binding of B19V to RBCs did not cause direct hemolysis but an increased OF of the cells. The increased fragility was not observed in the absence of calcium, which is required for PLA_2 activity (6), or in the presence of the PLA_2-specific antibody. It is interesting that although the PLA_2 antibody was able to fully protect the RBCs, the antibody could only moderately inhibit PLA_2 activity in an in vitro assay with a soluble PLA_2 substrate. This result likely indicates that the antibody protects the membrane by interpolating itself between the enzyme and the phospholipids of the cell membrane. The PLA_2 inhibitor manoalide was able to inhibit the B19V PLA_2 activity. However, the dose required for the inhibition (above 75 μM) was cytotoxic and caused the direct hemolysis of the RBCs (data not shown); therefore, it was not possible to use it in the OF test. Further studies would be required to verify whether the B19V-induced membrane damage also occurs during the natural infection.

B19V receptor globoside is the most abundant neutral glycolipid in the RBC membrane, with more than 10^7 antigens per cell (16). In our studies, the amount of capsid bound per cell is largely below this level. A possible explanation is that B19V binding to RBCs is complex and involves globoside in concert with other molecular structures. In agreement with this is the fact that B19V does not bind to membrane-associated globoside in vitro (19) and that an antibody specific for globoside was not able to totally block B19V binding to RBCs (Fig. 3B). Moreover, in our studies the amount of virus bound per cell ranged between 3,000 and 30,000 virions per cell. This significant variation exceeded that of globoside, whose concentration is rather similar among different individuals, regardless of whether their P blood group phenotype is P_1 or P_2 (16).

B19V viremia is always evaluated in the plasma fraction of the blood while the RBC fraction is systematically neglected. Cell-associated viremia is therefore unknown. The analysis of a blood sample from an individual with a recent virus infection revealed that at this particular moment of the infection, B19V was mostly associated to the RBC fraction. However, since B19V IgM- and IgG-specific antibodies were present, the result could reflect a faster elimination of B19V from plasma than from RBC-associated B19V. Therefore, in order to characterize cell-associated viremia and determine the extent of B19V binding to RBCs in a natural infection, more samples should be analyzed, particularly from early viremic blood samples without detectable B19V-specific antibodies. These results together highlight the importance of the human RBCs in the context of B19V infection.

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